# Phagocytosis of Fibronectin and Collagens Type I, III, and V by Human Gingival and Periodontal Ligament Fibroblasts In Vitro

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**Background:** Electron microscopic studies have suggested that the volume density of collagen-containing vacuoles in fibroblasts is higher in the periodontal ligament (PDL) than in the gingiva. Whether this difference reflects intrinsic differences in phagocytic capacity among the cells in these tissues is not known.

**Methods:** PDL and gingival fibroblasts were isolated from subjects and cultured under identical conditions in the presence of fluorescent beads coated with collagen type I, III, or V or fibronectin. Control beads were coated with bovine serum albumin or an enamel matrix protein mixture that does not constitute part of the extracellular matrix of PDL and gingiva. After various time intervals (1 to 24 hours), the percentage of cells that had internalized beads was assessed by flow cytometry. Since alkaline phosphatase activity has been suggested to play a role in collagen phagocytosis, the activity of this enzyme was determined for all cell populations.

**Results:** The results demonstrated the following order in the percentage of cells internalizing protein-coated beads: fibronectin > collagen type I > III > V. Internalization of collagen type I-coated beads exceeded that of beads coated with bovine serum albumin or enamel matrix proteins by 6 and 3 times, respectively. No differences were observed in collagen phagocytic activity between PDL and gingival fibroblasts, and no relationship could be demonstrated between collagen phagocytosis and alkaline phosphatase activity.

**Conclusions:** We conclude that differences in collagen phagocytosis between PDL and gingiva, as observed in vivo, are not likely to be explained in terms of intrinsic phagocytic capacities of these cells. *J Periodontol 2001;72:1340-1347.*

**KEY WORDS**

**Phagocytosis; collagen; periodontal ligament/physiology; gingiva/physiology; fibroblasts, gingival/physiology.**

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Under physiological conditions, fibroblasts degrade the collagenous matrix by which they are surrounded, primarily via a phagocytic pathway.[1] An electron microscopic study in rats showed that fibroblasts of the periodontal ligament (PDL) contain more phagocytosed collagen than gingival fibroblasts,[2] suggesting site-specific differences in phagocytic capacity. This could either result from intrinsic differences between the fibroblast populations or from influences of local tissue factors.

Differences in tissue architecture and composition between the PDL and gingiva might be related to distinct phagocytic activities of the fibroblasts harboring these tissues. Both PDL and gingiva consist of a heterotypic mixture of collagens,[3] non-collagenous proteins,[4] and proteoglycans.[5] Since phagocytosis of the various types of extracellular matrix molecules is likely to occur at different rates,[6] a distinct composition of the extracellular matrix may lead to differences in phagocytic activity. Cultured PDL fibroblasts appear to synthesize more collagen and fibronectin than gingival fibroblasts.[7-9]

Functional differences between the 2 soft connective tissues may also modulate their phagocytic activities. Whereas the gingiva is connected only to cementum, the PDL is positioned between 2 mineralized tissues, cementum and bone. This implies distinct patterns of shock absorption among the 2 tissues. Recent studies have shown that PDL fibroblasts respond to very weak forces with the release of nitric oxide (NO), whereas gingival fibroblasts do not.[10] This second messenger molecule has been proposed to play a role in tissue remodeling.[11-14] Moreover, mechanical stress alters the pattern of extracellular matrix synthesis by both PDL and gingival fibroblasts in vitro,[15,16] suggesting that this factor may also modulate matrix turnover.

The aim of the present study was to test the hypothesis that differences in collagen phagocytosis between PDL and gingiva are due to intrinsic differences between the fibroblast populations harboring these tissues. To this end, fibroblasts were isolated from human PDL and gingiva and cultured under identical conditions in the presence of fluorescent beads coated with collagen type I, III, or V, fibronectin, or the control proteins, bovine serum albumin or enamel matrix proteins.

## MATERIALS AND METHODS

Dulbecco's modified eagle medium (DMEM), fetal calf serum (FCS), Hanks' balanced salt solution (HBSS), and trypsin were obtained from Gibco.[†] Penicillin, streptomycin, amphotericin B, bovine serum albumin (BSA, fraction V), para-nitrophenylphosphate (pNPP), cytochalasin B, purified human placenta type V collagen, fluorescein isothiocyanate (FITC)-labeled sheep anti-mouse (IgG fraction), FITC-labeled goat anti-rabbit (IgG fraction), tetramethylrhodamine isothiocyanate (TRITC)-labeled rabbit anti-goat (IgG fraction), TRITC-labeled goat anti-rabbit (IgG fraction) and mouse IgG were purchased from Sigma.[§] Dimethylsulfoxide (DMSO) was from Pierce.[‖] Picogreen assay was from Molecular Probes.[¶] Culture flasks and 6- and 24-well plates were from Costar.[#] Yellow green microspheres (beads, 2.0 μm) were from Polysciences Inc.[**] Human leiomeiyoma collagen type I was isolated and purified according to the method of ChandraRajan.[17] Human collagen type III and goat monoclonal anti-human collagen type I (IgG fraction) were obtained from Southern Biotechnology Associates Inc.[††] Purified serum fibronectin was from Boehringer Mannheim.[‡‡] Enamel matrix proteins (EMP) were a gift from Biora AB.[§§] Human keratinocytes were a gift from Dr. A. van den Bogaerdt (Department of Dermatology, Academic Medical Center, Amsterdam). A mouse monoclonal antibody, ASO2 (IgG fraction), to a human fibroblast specific antigen located on the cell surface, was purchased from Dianova.[‖‖] Rabbit polyclonal pancytokeratin (IgG fraction) was obtained from Zymed.[¶¶] A rabbit polyclonal antibody directed against bovine amelogenin, raised by Dr. J.D. Termine (National Institutes of Health, Bethesda, Maryland), was provided by Dr. A.L.J.J. Bronckers (Department of Oral Cell Biology, Academic Center for Dentistry, Amsterdam). Goat and rabbit IgG were isolated and purified from serum by using ammoniumpersulphate precipitation. Flow cytometry analysis was performed by using a Facstar Plus flow cytometer.[##]

### *Cell Isolation and Culture*

Fibroblasts were obtained from the vestibular free gingiva and from the PDL of extracted premolars or third molars of healthy individuals (aged 13 to 28), without overt clinical signs of inflammation in the periodontal tissues (no visible plaque, periodontal probing depth <3 mm, no bleeding on gentle probing, no signs of clinical attachment loss). Prior to extraction, informed consent was obtained. The fibroblasts were obtained and cultured as described previously by van der Pauw et al.[18] Gingival and PDL fibroblasts from each subject were cultured under identical conditions. In brief, the extracted teeth and the gingival biopsies were washed twice in DMEM supplemented with 10% FCS, penicillin (1,000 units/ml), streptomycin (0.1 mg/ml), and amphotericin B (2.5 μg/ml). The PDL attached to the middle third of the roots was cut from the root surface by means of a scalpel knife. The PDL and gingival biopsies were then cut into small pieces and placed in 6-well culture plates and incubated with 1.5 ml DMEM supplemented with 10% FCS and penicillin (100 units/ml), streptomycin (10 μg/ml), and amphotericin B (0.25 μg/ml) in a humidified atmosphere of 5% $CO_2$ in air at 37°C. After a culture period of 5 to 8 weeks, cells surrounding the tissue explants were confluent and collected by use of 0.25% trypsin and 0.1% ethylenediamine tetraacetic acid (EDTA) (pH 7.3), transferred to 25 $cm^2$ culture flasks, and designated as passage 1 (P1). The cells of P1 and subsequent passages showed a typical fibroblastic shape when confluent. Immunolocalization with a specific antifibroblast antibody (ASO2) revealed that all cells stained positive for this fibroblast marker. The presence of epithelial cells was examined with an antibody directed against cytokeratin. Human keratinocytes served as a positive control. Cells maintained in culture from PDL and gingiva were all negative for cytokeratin. For all experiments, cells between the third and seventh subculture were used. Phagocytic activity of PDL and gingival fibroblasts was compared when these populations were in the same subculture.

### *Coating of Beads*

Coating of the beads was performed according to the method described by McKeown and coworkers.[19] In short, for coating with collagen type I, III, or V, 5 μl of the bead stock solution was incubated with 3 mg/ml

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‡‡ Boehringer Mannheim, Mannheim, Germany.
§§ Biora AB, Malmö, Sweden.
‖‖ Dianova, Hamburg, Germany.
¶¶ Zymed, Zymed Lab. Inc., San Francisco, CA.
\## Becton Dickinson, Mississauga, ON.

collagen (dissolved in 0.01 M acetic acid) overnight under continuous stirring. Incubated beads were centrifuged for 2 minutes at 10,000 rpm. Five hundred (500) µl Tris/HCl buffer (pH 7.0) was added to the beads to induce polymerization of the collagen. Coated beads were then centrifuged for 2 minutes at 10,000 rpm and washed with 1 ml of phosphate buffered saline (PBS). In addition, beads were coated with BSA, EMP, or fibronectin. To this end, 3 mg/ml BSA (dissolved in PBS), EMP (dissolved in 0.01 M acetic acid), or fibronectin (dissolved in PBS) was added to 5 µl bead stock solution. Beads were incubated overnight under continuous stirring, centrifuged, and washed with 1 ml of PBS.

In a preliminary series of experiments, we analyzed by immunolocalization whether the coating procedures were efficient. Collagen type I- and EMP-coated beads were incubated with a goat monoclonal antibody to human collagen type I or a rabbit polyclonal antibody to bovine amelogenin, respectively. After incubation with the second antibodies (TRITC-labeled rabbit anti-goat and TRITC-labeled goat anti-rabbit IgG), immunofluorescent analysis revealed that the beads were coated with their respective proteins.

### *Phagocytosis Assay*

To study phagocytosis of coated beads by PDL and gingival fibroblasts, cells were seeded in 6-well ($4.0 \times 10^4$ cells/well) or 24-well plates ($7.5 \times 10^3$ cells/well) in, respectively, 2 ml and 300 µl DMEM supplemented with 10% FCS and antibiotics and cultured for 1 day. The medium was changed for DMEM with 5% FCS and antibiotics, and the cells were kept in this medium for another 24 hours. This was followed by 2 days of serum-free incubation in DMEM supplemented with BSA (4 mg/ml). Prior to use, coated beads were sonicated 3 times for 5 seconds to disperse bead clumps and vortexed. Bead concentration was determined by using a Bürker-Türker counter. A minimum of 5 beads per cell in DMEM supplemented with BSA and antibiotics were added to each well.

To interfere with polymerization of cytoplasmic microfilaments, cytochalasin B was added to a series of wells in a concentration of 2.0 µg/ml or DMSO as a control. Following incubation, the cells were treated as indicated above. After an incubation period of 1, 3, 6, or 24 hours, cells were trypsinized with 0.25% trypsin and 0.1% EDTA in PBS for 8 to 10 minutes. By using trypsin, non-internalized beads were removed from the cell membrane.[19,20] The cells were washed with DMEM supplemented with BSA and centrifuged for 5 minutes at 1,500 rpm. For flow cytometry analyses, the cell pellet was dissolved in 500 µl 1% BSA in PBS, vortexed, and stored until analysis at 4°C.

In order to determine cell viability, cells were incubated with eosin after 1, 3, 6, or 24 hours of culturing with coated beads. Cell counting revealed that over 90% of the cells were viable under the conditions of this study.

### *Flow Cytometry Analysis*

Cells were analyzed by flow cytometry with 488 nm excitation (laser power 150 mW) and a 530 ± 15 nm band-pass filter (FL1) in the emission path. Only cells with forward and side scatter characteristics similar to those of whole intact cells were included in the analysis. For all flow cytometry analyses, at least $5 \times 10^3$ cells of each sample were assayed.

### *Alkaline Phosphatase Activity*

To assess alkaline phosphatase (ALP) activity, the medium of cultured cells was removed and 200 µl 0.1% Triton-X-100 in diethanolamine buffer (1 mol/l diethanolamine, 0.5 mmol/l $MgCl_2$ pH 9.8) was added to each well. The culture plates were stored at –20°C. Cell extracts were collected after thawing and an overnight incubation at 4°C while shaking continuously. To 10 µl cell extract, pNPP (final concentration 6 mmol/l) in 200 µl diethanolamine buffer was added as a substrate and incubated at 37°C. The extinction was read directly after addition of the substrate and after 2, 6, and 16 hours of incubation using a microplate reader*** at 405 nm. Each sample was assayed in triplicate. Enzyme activity was expressed in units, with one unit defined as 1 µmol/l para-nitrophenol released per minute (at 37°C and pH 9.8). Enzyme activity was presented per µg DNA (see DNA assay).

### *DNA Assay*

DNA analysis was performed on 10 µl cell extract using the picogreen assay according to the manufacturer's instructions.

### *Transmission Electron Microscopy*

After culturing PDL and gingival fibroblasts for 24 hours in the presence of collagen type I-coated beads, the medium was discarded and cells were fixed for 24 hours at room temperature in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The cells were then washed, postfixed in 1% $OsO_4$ in 0.1 M sodium cacodylate buffer, washed in buffer, dehydrated through a graded series of ethanol, and embedded in epoxy resin.

Semithin sections were made with glass knives parallel to the surface of the culture plate. The sections were stained with methylene blue, and areas with fibroblasts were selected. Ultrathin sections were cut from these areas, stained with lead and uranyl, and examined in an electron microscope.†††

*** Wallac, Turku, Finland.
††† Model EM10C, Carl Zeiss B.V., Weesp, The Netherlands.

### Data Analysis

Statistical analysis was performed by using analysis of variance (ANOVA) and Student's $t$ test. Differences were considered significant when $P<0.05$ (2-tailed).

## RESULTS

### Electron Microscopy

In order to analyze whether beads were internalized by the fibroblasts, cells were incubated for 24 hours in the presence of collagen type I-coated beads and examined in the electron microscope. The beads were found within vacuolar structures in the cytoplasm and proved to be surrounded by a membrane (Fig. 1). The space between the beads and membrane was filled with electron translucent material. The internalized beads proved to be present not only in the peripheral part of the cells, but also in the more central portion adjacent to the nucleus.

### Quantitative Evaluation

**Phagocytosis of collagen type I versus BSA, EMP.** Both PDL and gingival fibroblasts showed a time-dependent increase of the number of internalized beads coated with collagen type I, BSA, or EMP (Fig. 2). During the first 6 hours of culturing, the percentage of cells positive for coated beads showed a rapid increase, which tended to level off after 24 hours. Compared with uptake of beads coated with the control proteins BSA or EMP, a statistically significant higher percentage of cells (38%) had internalized collagen type I-coated beads (Fig. 2A). PDL and gingival fibroblasts did not show significant differences in the internalization of collagen type I-coated beads.

A very low percentage of cells (≤6%) proved to endocytose BSA-coated beads (Fig. 2B), whereas a somewhat higher percentage of cells (13%) had internalized EMP-coated beads. This was the case for both PDL and gingival fibroblasts. With respect to the BSA- and EMP-coated beads, no significant differences were

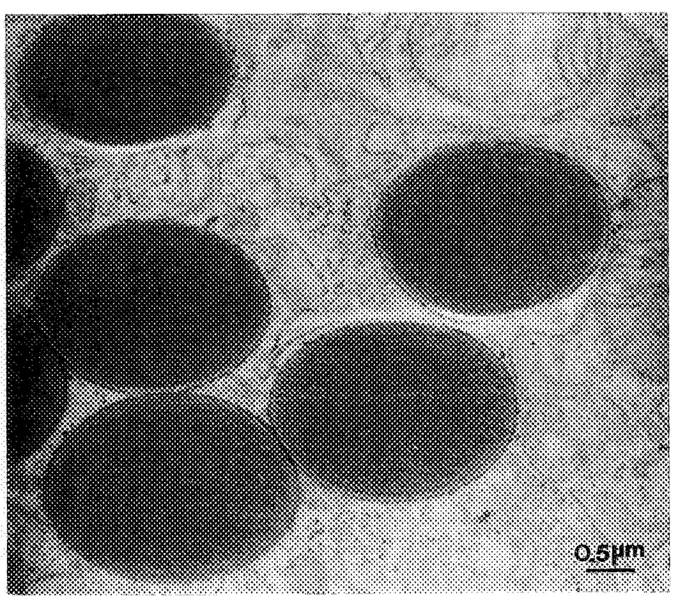


***Figure 1.***
*Electron micrograph of a PDL fibroblast cultured for 24 hours in the presence of beads coated with collagen type I (original magnification ×15,000).*

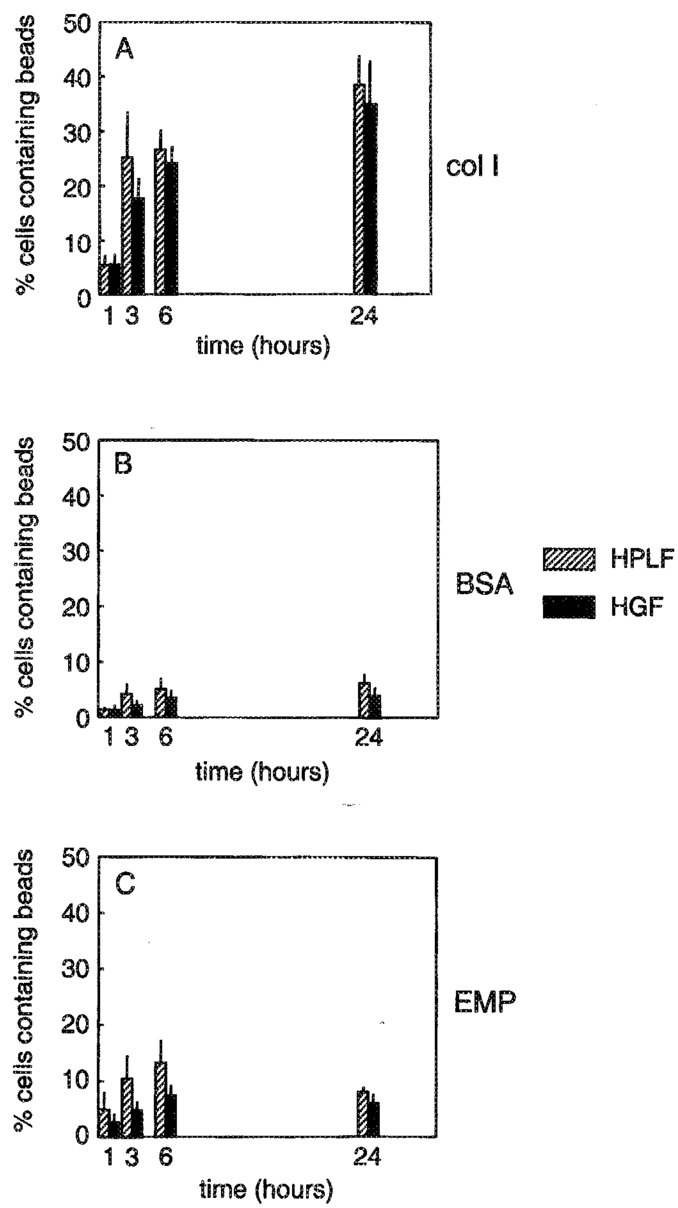


***Figure 2.***
*Percentage of PDL and gingival fibroblasts containing beads coated with collagen type I* ***(A)****, BSA* ***(B)****, and EMP* ***(C)*** *after 1, 3, 6, and 24 hours of culturing. Fibroblasts were obtained from 4 to 6 subjects. Data are given as mean ± SD. Statistical analysis was conducted by analysis of variance: col I versus BSA: P <0.01; col I versus EMP: P <0.05; EMP versus BSA: not significant. PDL versus gingival fibroblasts, BSA, EMP, col I: not significant.*

found between PDL and gingival fibroblasts (Figs. 2B and 2C).

In order to analyze the number of internalized collagen type I-coated beads per cell, the cells were categorized into one of the following groups: cells with 1 bead, 2 beads, or 3 or more beads (Fig. 3). Most cells contained 1 coated bead only. With time, an increasing percentage of cells with internalized beads was noted for all 3 groups.

**Differences in phagocytosis of collagen type I, III, V, and fibronectin.** No differences were found between PDL and gingival fibroblasts in the phagocytosis of beads coated with the different types of collagen and fibronectin. Therefore, the data of the 2 fibroblast populations were combined and are presented together.

Considerable differences were observed in the percentage of cells internalizing the different types of collagen and fibronectin (Fig. 4). Fibronectin-coated beads were internalized in greater numbers than beads coated with collagen types I, III, and V. Collagen type I beads were internalized to a significantly higher extent than type V beads. No significant difference was observed between uptake of beads coated with collagen type I and III and between types III and V.

**Cytochalasin B.** To investigate the involvement of cytoplasmic microfilaments in the process of internalization,[19,21] cytochalasin B was added to the cultures. Cells cultured for 3 hours in the presence of 2.0 μg/ml cytochalasin B spread to a lesser extent than control cells. The drug proved to inhibit the internalization of collagen type I-coated beads by both fibroblast populations (Fig. 5). Inhibition of internalization was approximately 40%. Endocytosis of BSA-coated beads by PDL and gingival fibroblasts was not influenced by cytochalasin B.

### *ALP Activity and Collagen Phagocytosis*

Since ALP has been suggested to be involved in the internalization of collagen-coated beads,[22] we assessed the activity of this enzyme in the 2 fibroblast populations. PDL fibroblasts expressed significantly higher levels of ALP activity than gingival fibroblasts (PDL: 0.26 ± 0.07 mU/μg DNA; gingiva: 0.07 ± 0.04 mU/μg DNA; $P$ <0.05). No relationship, however, was found between the expression of ALP activity and the percentage of fibroblasts that had phagocytosed collagen-coated beads (r = 0.19, df = 10, NS). Also, no relationships were found between the enzyme activity and the percentage of cells that had internalized BSA- or EMP-coated beads.

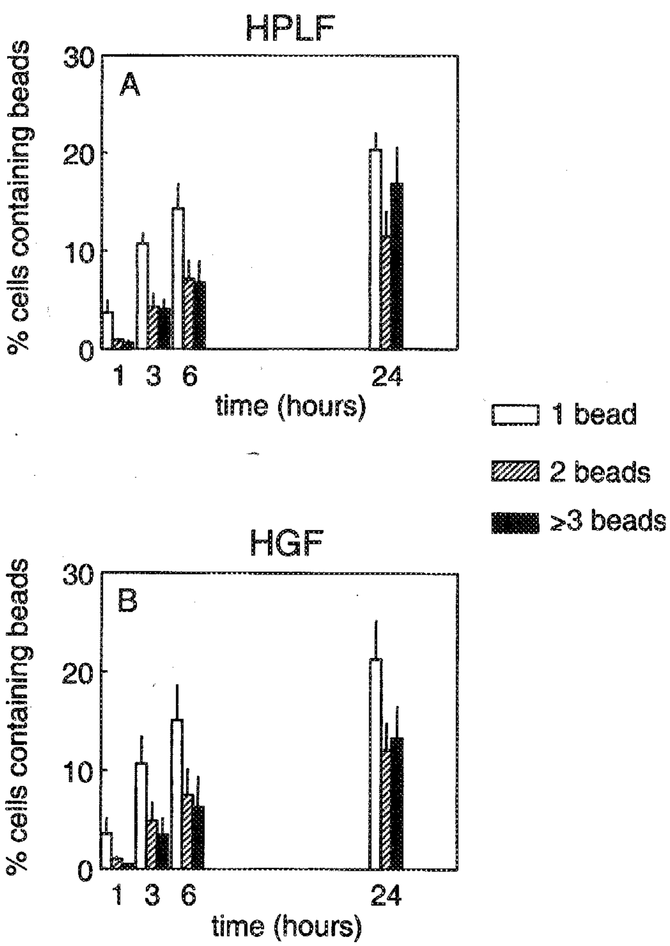


***Figure 3.***
*The percentage of PDL **(A)** and gingival **(B)** fibroblasts containing collagen type I-coated beads divided in groups positive for 1, 2, or ≥ 3 beads. Data are presented as mean ± SD. Fibroblasts were obtained from 4 subjects.*

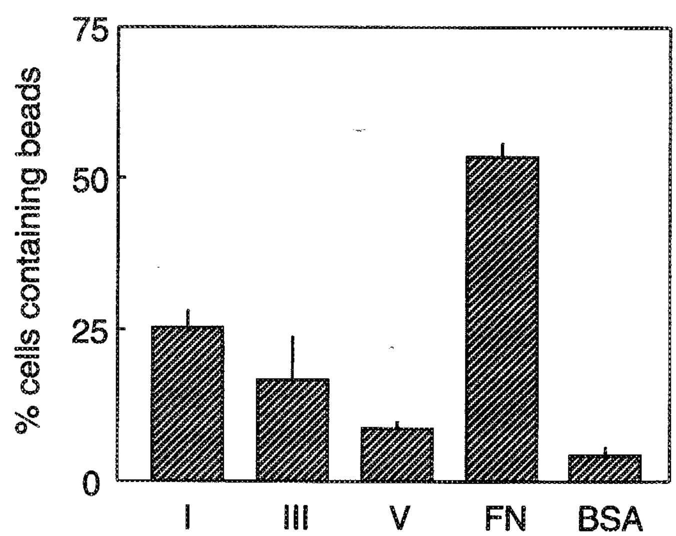


***Figure 4.***
*Percentage of fibroblasts containing beads coated with collagen type I, III, and V; fibronectin (FN); and BSA after 6 hours of culturing. Data are presented as mean ± SD. Fibroblasts were obtained from 2 to 6 subjects. Statistical analysis was conducted by ANOVA: col I versus col III: not significant; col I versus col V: P <0.01; col I versus FN: P <0.001; col III versus FN: P <0.001; col V versus FN: P <0.001; col III versus col V: not significant.*

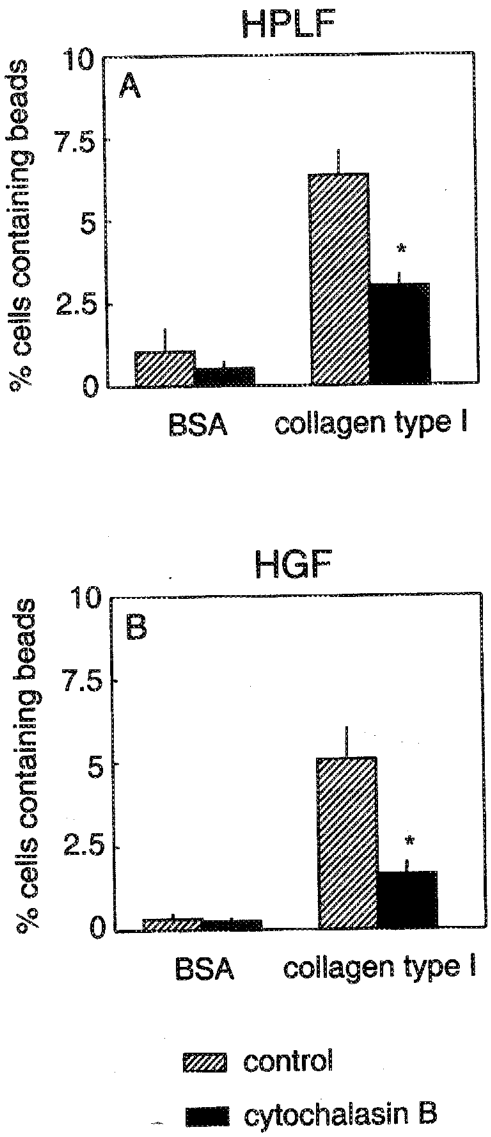


***Figure 5.***
*PDL **(A)** and gingival **(B)** fibroblasts incubated for 3 hours with beads coated with BSA or collagen type I in the absence or presence of 2.0 µg/ml cytochalasin B. Data are presented as mean ± SD (n = 5). Fibroblasts were obtained from 1 subject. Statistical analysis was done by ANOVA. PDL and gingival fibroblasts: BSA versus col I: P <0.001; col I: control versus cytochalasin B: P <0.001.*

## DISCUSSION

The present data have demonstrated that fibroblasts from both human PDL and gingiva phagocytose collagen-coated beads. Since uptake of beads could be inhibited by cytochalasin B, our findings indicate that the internalization depends on an intact microfilament apparatus. In agreement with others,[6] we observed large differences between the internalization of beads coated with the different types of extracellular matrix proteins. These findings indicate that internalization of protein-coated beads is not a random process governed by physical factors, but depends to a large extent on the biochemical nature of the coatings. Extracellular matrix components like fibronectin and collagens evoked a distinct phagocytic response of the cells, whereas proteins not constituting part of the normal connective tissue matrix did not, or did so to a lesser extent.

A striking finding was the absence of any significant difference in the uptake of collagen- and fibronectin-coated beads between PDL and gingival fibroblasts. In addition, no relationship was found between collagen phagocytosis and the expression of alkaline phosphatase (ALP) activity. Our data would seem to contradict previous reports on differences in collagen phagocytosis in vivo between cells in PDL and those in gingiva.[2] We, therefore, tend to conclude that the differences as observed in vivo do not relate to intrinsic differences in phagocytic capacity, but are likely due to local tissue factors, related either to the specific architecture of the tissues and/or to local functional influences. Interestingly, fibroblasts cultured from PDL and gingiva have been shown to respond differently to forces applied in an in vitro model.[10] In contrast to gingival fibroblasts, PDL fibroblasts release nitric oxide (NO) in response to a fluid flow exerted upon the cells. NO is known to have widespread effects on connective tissue metabolism.[12,23,24] Moreover, this second messenger has been suggested to be involved in the maintenance of the PDL space.[25] Despite the fact that the precise actions of NO in periodontal tissues are still unknown, this molecule might play a role in the modulation of collagen phagocytosis.

Since we have shown differences between the phagocytosis of beads coated with collagen type I, III, and V and fibronectin, differences in the structural organization and composition of the extracellular matrix of PDL and gingiva in vivo may have their own influence on the remodeling rate. Both PDL and gingiva consist of a heterotypic mixture of collagen, including types I, III, IV, V, and VI, fibronectin, and proteoglycans.[3,4,26] PDL fibroblasts have been shown to synthesize higher levels of fibronectin and collagen in vitro than gingival fibroblasts.[7-9] Thus, it is not unlikely that in vivo the amounts of the various extracellular matrix components differ between the two tissues. Interestingly, fibronectin, which coats collagen fibrils in vivo,[27] has been proposed to initiate phagocytosis by acting as a recognition site for fibroblasts.[19] Since we found that fibroblasts phagocytose fibronectin-coated beads more rapidly than collagen type I beads, a higher level of fibronectin in the PDL might result in increased phagocytic activity.

Furthermore, growth factors and cytokines within the tissue may influence phagocytosis of matrix constituents. It is known that transforming growth factor-β (TGF-β) upregulates, whereas interleukin-1 downregulates collagen phagocytosis by fibroblasts.[28] One of the effects of TGF-β may be the modulation of matrix metalloproteinase (MMP) activity, in particular MMP-2.[29] Selective inhibition of this enzyme almost completely blocks collagen degradation.[30] Moreover, a significant correlation has been found between the remodeling rate of soft connective tissues (PDL, gingiva, and skin) and the amount of active MMP-2.[30] MMP-13, which is expressed by oral gingival epithelium[31] and gingival fibroblasts,[32] might also be involved in periodontal soft connective tissue remodeling. Since MMP-13 degrades a wide range of ECM components, including types I and III collagen, tenascin, and fibronectin,[33] we hypothesize that this enzyme is particularly expressed at sites where a rapid remodeling activity is required.

Significant differences were observed in the internalization of EMP- and BSA-coated beads by PDL and gingival fibroblasts. In addition to receptor-ligand interactions, physicochemical properties could play a role in the internalization of these proteins. Hydrophobic microorganisms, for instance, are phagocytosed in higher amounts by macrophages than hydrophilic strains.[34] Since EMP is a hydrophobic protein mixture[35] and albumin shows hydrophilic properties,[36,37] the differences in internalization between these 2 types of proteins might (in part) be due to their distinct wettabilities.

A study by Hui et al. would seem to suggest that membrane-bound ALP dimers play a role in the process of collagen phagocytosis by fibroblasts in vitro.[22] These authors studied cells that did or did not express ALP and showed that cells expressing this enzyme phagocytosed more collagen-coated beads. Interestingly, PDL fibroblasts are known to express higher levels of ALP activity than gingival fibroblasts.[7,38] Thus, it was to be expected that PDL fibroblasts would phagocytose more collagen than gingival fibroblasts. The present study, however, has shown that this is not the case: both populations showed similar phagocytic activities, suggesting that ALP is not essential for phagocytosis. In line with this are recent data presented by Beertsen et al.,[39] who demonstrated that in mice deficient for tissue non-specific ALP, tooth eruption and collagen remodeling appeared to proceed more or less normally.

In conclusion, our results demonstrate that PDL and gingival fibroblasts have similar affinities to collagen-coated beads and internalize them at about the same rate. We propose that differences in collagen phagocytosis between the cells in their respective natural domains are likely the result of differences in tissue composition or external functional influences.

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